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Characterization of meticillin-resistant and meticillin-susceptible isolates of *Staphylococcus pseudintermedius* from cases of canine pyoderma in Australia

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Meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) has recently emerged as a worldwide cause of canine pyoderma. In this study, we characterized 22 *S. pseudintermedius* isolates cultured from 19 dogs with pyoderma that attended a veterinary dermatology referral clinic in Australia in 2011 and 2012. Twelve isolates were identified as MRSP by *mecA* real-time PCR and phenotypic resistance to oxacillin. In addition to β-lactam resistance, MRSP isolates were resistant to erythromycin (91.6 %), gentamicin (83.3 %), ciprofloxacin (83.3 %), chloramphenicol (75 %), clindamycin (66 %), oxytetracycline (66 %) and tetracycline (50 %), as shown by disc-diffusion susceptibility testing. Meticillin-susceptible *S. pseudintermedius* isolates only showed resistance to penicillin/ampicillin (90 %) and tetracycline (10 %). PFGE using the *Sma*I restriction enzyme was unable to type nine of the 12 MRSP isolates. However the nine isolates provided the same PFGE pulsotype using the *Cfr*91 restriction enzyme. Application of the *mec*-associated direct repeat unit (*dru*) typing method identified the nine *Sma*I PFGE-untypable isolates as dt11cb, a *dru* type that has only previously been associated with MRSP sequence type (ST)45 isolates that possess a unique SCC*mec* element. The dt11cb isolates shared a similar multidrug-resistant antibiogram phenotype profile, whereas the other MRSP isolates, dt11a, dt11af (dt11a-associated) and dt10h, were resistant to fewer antibiotic classes and had distinct PFGE profiles. This is the first report of MRSP causing pyoderma in dogs from Australia. The rapid intercontinental emergence and spread of multidrug-resistant MRSP strains confirms the urgent need for new treatment modalities for recurrent canine pyoderma in veterinary practice.

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INTRODUCTION

Meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) is an emerging pathogen in veterinary companion-animal practice, affecting dogs, cats and horses (Morris *et al.*, 2010; Bannoehr & Guardabassi, 2012). Furthermore, there is evidence of MRSP transmission to owners and veterinary personnel caring for infected pets (Morris *et al.*, 2010; Paul *et al.*, 2011; van Duijkeren *et al.*, 2011), leading to concerns that MRSP could adapt to become a resident commensal organism in humans, with subsequent horizontal transmission between individuals (Weese, 2012).

Abbreviations: *dru*, direct repeat unit; MRSP, meticillin-resistant *Staphylococcus pseudintermedius*; MSSP, meticillin-susceptible *Staphylococcus pseudintermedius*; ST, sequence type.

The two most commonly reported MRSP clones in the literature are multilocus sequence type (ST)71, which is reported to be dominant among European and Japanese MRSP isolates, and ST68, which appears to have a comparatively higher prevalence in North America (Perreten *et al.*, 2010; Bardiau *et al.*, 2013). A new MRSP subtype belonging to clonal complex 179 and ST45 has also recently been identified in dogs from Israel and Thailand (Perreten *et al.*, 2013).

Despite being contained within a mobile genetic element, sequence analysis of the *mec*-associated direct repeat unit (*dru* typing) has been recently described as a simple, rapid and cost-effective technique for subtyping meticillin-resistant staphylococci (Goering *et al.*, 2008). In addition, MRSP *dru* typing has recently shown that MRSP ST71 and ST68 are predominantly associated with *dru* clusters 9a and 11a, respectively (Weese *et al.*, 2013).

Although several studies have confirmed the presence of meticillin-resistant *Staphylococcus aureus* in dogs and cats (Malik *et al.*, 2006), veterinary personnel (Jordan *et al.*, 2011) and horses (Axon *et al.*, 2011) in Australia, MRSP has not been described previously in Australian companion animals. However, as Australia does not have a coordinated antimicrobial-resistance surveillance programme focused on either companion-animal or livestock isolates, identification of MRSP strains, including those with a multidrug-resistant phenotype, may be under-reported.

In this study, we report the isolation of MRSP strains from dogs with pyoderma referred to a veterinary dermatology clinic in Perth, Western Australia, and their preliminary characterization.

METHODS

Bacterial strains and identification. Pyoderma samples were aseptically collected from 19 dogs of various ages, sexes and dermatologic conditions that attended the Animal Dermatology Clinic, Perth, Western Australia, between February 2011 and November 2012. The dogs were diagnosed with either superficial or deep bacterial pyoderma that had not responded to empirically selected systemic antibiotics (White, 1996). A diagnosis of pyoderma was made if the dog had consistent clinical signs including papules, pustules, crusted papules, epidermal collarettes, nodules or draining tracts, and/or cytological evidence of bacteria. Overall, 171 aseptically collected skin samples (skin biopsy, swab or fine-needle aspirate) were sent to a private diagnostic laboratory for culture and susceptibility testing.

Isolates were identified as *S. pseudintermedius* on the basis of exhibiting double-zoned haemolysis on sheep blood agar, growth on mannitol salt agar, a positive reaction to the tube coagulase and pyrrolidonyl arylamidase tests and a negative reaction for the production of acetoin on the Voges–Proskauer test. Identification was confirmed by Vitek mass spectrometry (matrix-assisted laser desorption/ionization time of flight).

Antibiogram phenotyping. Antimicrobial susceptibility profiles were determined by disk diffusion according to Clinical Laboratory Standards Institute criteria (CLSI, 2008, 2013). The following antimicrobials were included: penicillin (10 units), ampicillin (10 μg), amoxicillin (30 μg), oxacillin (1 μg), cephalothin (20 μg), cefotetan (30 μg), erythromycin (15 μg), clindamycin (2 μg), gentamicin (10 μg), chloramphenicol (10 μg), tetracycline (30 μg), oxytetracycline (30 μg), ciprofloxacin (5 μg), moxifloxacin (5 μg) and rifampicin (5 μg). Oxacillin MICs were determined using Etest strips (bioMérieux). Resistance scores were calculated for each isolate as the cumulative number of resistance phenotypes for the nine tested non-β-lactam antimicrobials.

Screening for *mecA* and PFGE. The *mecA* gene was detected by real-time PCR, as described previously (Costa *et al.*, 2005). Genetic relatedness of the isolates was determined by PFGE using *Sma*I (Roche) and *Cfr*91 (Thermo Scientific) restriction enzymes, as described previously (Perreten *et al.*, 2013). The pulse times were 5–40 s over 18 h and 20–25 s over 5 h. Chromosomal patterns were examined visually, scanned with a Quantity One device (Bio-Rad Laboratories) and digitally analysed using FPQuest (Applied Maths). The Dice coefficient and the unweighted pair group method with arithmetic mean were used with settings for tolerance and optimization of 1.25 and 0.5 %, respectively. Isolates with 80 % or greater similarity were considered to be the same pulsotype.

***dru* typing.** Sequence analysis of the *mec*-associated *dru* region was performed on all *mecA*-positive isolates, as described previously (Goering *et al.*, 2008). Cluster analysis of *dru* sequences was performed using the polymorphic variable number tandem repeat plug-in tool of the BioNumerics software program (version 6.6; Applied Maths). A minimum spanning tree was generated from the similarity matrix using BioNumerics with the root node assigned to the ST with the greatest number of related types. Distance intervals were created using a bin distance of 1.0 %. *dru* types separated by a multispacer ST distance of less than 1 (>98 % similarity) were considered closely related and assigned to the same cluster.

RESULTS

Antimicrobial-resistance phenotypes and *mecA* status

Twelve *Staphylococcus* isolates (7 % of total swabs or tissue biopsies submitted for culture and susceptibility during the study period; Table 1) harboured the *mecA* gene and had oxacillin MICs ranging from 1.5 to >256 μg ml^{-1}, confirming their identification as MRSP. Between August 2012 and November 2012, the first 10 *S. pseudintermedius* isolates showing *in vitro* susceptibility to two of three β-lactam antibiotics (cephalexin, amoxicillin or amoxicillin–clavulanic acid) were selected for comparison with the MRSP isolates. These 10 isolates were confirmed as meticillin-susceptible *S. pseudintermedius* (MSSP), based on the oxacillin MIC (0.125–0.25 μg ml^{-1}) and a negative *mecA* PCR result. Thus, a total of 22 *S. pseudintermedius* isolates (16 swabs, six skin biopsies) from 19 dogs were further characterized. Signalment, site of collection, sample type and the clinical history of each isolate are shown in Table 1.

The MRSP isolates were resistant to erythromycin (91.7 %), gentamicin (83.3 %), ciprofloxacin (83.3 %), chloramphenicol (75 %), clindamycin (66.7 %), oxytetracycline (66.7%) and tetracycline (50 %) (Table 2). By contrast, the MSSP isolates were primarily resistant to only penicillin and ampicillin (both 90 %), with only one isolate resistant to tetracycline.

Table 1. Signalment, site of collection, collection technique and underlying primary disease associated with the 12 MRSP and 10 MSSP isolates obtained from dogs with pyoderma in Australia

Isolate	Date of isolation	Age	Sex	Breed	Site of collection	Collection technique	Underlying primary disease
MRSP							
SP1	17 February 2011	3 years, 4 months	MN	Mastiff cross	Trunk	Swab	Atopic dermatitis
SP2*	22 February 2011	14 years, 5 months	FS	Shar pei cross	Foot	Tissue biopsy	Atopic dermatitis, fibroadnexal dysplasia
SP3	3 August 2011	7 years, 11 months	MN	Cavalier King Charles spaniel	Foot	Swab	Atopic dermatitis
SP4	3 August 2011	10 years, 1 month	MN	Shar pei cross	Trunk	Swab	Atopic dermatitis, adverse food reaction
SP5	25 August 2011	10 years, 3 months	MN	Miniature dachshund	Foot	Swab	Atopic dermatitis, adverse food reactions
SP6†	25 August 2011	9 years, 1 month	MN	British bulldog	Foot	Swab	Pemphigus foliaceus
SP7†	25 August 2011	9 years, 1 month	MN	British bulldog	Foot	Swab	Pemphigus foliaceus
SP8‡	27 June 2012	11 years, 3 months	FS	Akita	Trunk	Swab	Atopic dermatitis, polycystic ovaries
SP9‡	17 October 2012	11 years, 6 months	FS	Akita	Trunk	Swab	Atopic dermatitis, polycystic ovaries
SP10	3 October 2012	8 months	MN	Bull terrier	Trunk	Tissue biopsy	Adverse food reaction, cutaneous papillomatosis
SP11	11 October 2012	2 years, 3 months	MN	Great dane	Trunk	Swab	Atopic dermatitis
SP12	20 November 2012	9 months	FE	Dogue de Bordeaux	Trunk	Swab	Atopic dermatitis
MSSP							
SP13	24 August 2012	7 years, 1 month	MN	Labrador retriever	Trunk	Tissue biopsy	Atopic dermatitis, cutaneous mast-cell tumour
SP14	12 October 2012	6 years	FS	Shih tzu cross	Trunk	Tissue biopsy	Pemphigus foliaceus
SP15	16 October 2012	4 year, 1 month	MN	Cavalier King Charles spaniel	Right external ear canal	Swab	Atopic dermatitis
SP16	1 November 2012	14 years	MN	Maltese	Trunk	Swab	Atopic dermatitis, pituitary-dependent hyperadrenocorticism
SP17	1 November 2012	7 years	MN	Labrador retriever	Right external ear canal	Swab	Aural inflammatory polyp
SP18	1 November 2012	12 years, 5 months	MN	Maltese	Trunk	Tissue biopsy	Atopic dermatitis, adverse food reactions, cutaneous squamous-cell carcinoma
SP19	2 November 2012	9 years, 9 months	FS	Jack Russell terrier	Trunk	Tissue biopsy	Atopic dermatitis, adverse food reaction
SP20	2 November 2012	1 year, 3 months	FS	Fox terrier	Trunk	Swab	Atopic dermatitis
SP21	8 November 2012	11 year, 4 months	ME	Labrador retriever	Foot	Swab	Atopic dermatitis
SP22*	28 November 2012	15 years, 4 months	FS	Shar pei cross	Trunk	Swab	Atopic dermatitis, fibroadnexal dysplasia

FE, Female entire; FS, female spayed; ME, male entire; MN, male neutered.

*Samples were taken from the same animal, 21 months apart.

†Samples were taken from different sites in the same animal.

‡Samples were taken from the same animal, 4 months apart.

Table 2. Antimicrobial susceptibility profiles, PFGE pulsotypes and *dru* types for the 22 *S. pseudintermedius* isolates obtained from dogs with canine pyoderma in Australia

Isolate	Pen	Amp	Amx	Oxa	Cef	Ctt	Ery	Cli	Gen	Chl	Tet	Ote	Cip	Mxf	Rif	RS	PFGE	*dru*	dct
MRSP																			
SP1	R	R	R	R	S	R	R	R	R	R	I	R	R	S	S	6	K	dt11cb	11a
SP2*	R	R	R	R	S	S	R	I	R	R	S	S	R	I	S	4	K	dt11cb	11a
SP3	R	R	R	R	I	S	R	R	R	R	R	R	R	S	S	7	K	dt11cb	11a
SP4	R	R	R	R	S	S	R	R	R	R	R	R	R	S	S	7	K	dt11cb	11a
SP5	R	R	R	R	S	S	R	R	R	R	I	R	R	S	S	6	K	dt11cb	11a
SP6†	R	R	R	R	R	S	R	R	R	R	R	R	R	S	S	7	K	dt11cb	11a
SP7†	R	R	R	R	I	S	R	R	R	R	R	R	R	S	S	7	K	dt11cb	11a
SP8‡	R	R	S	R	S	S	R	R	R	R	R	I	R	S	S	6	K	dt11cb	11a
SP9‡	R	R	R	R	S	S	R	R	R	R	I	R	R	S	S	6	K	dt11cb	11a
SP10	R	R	R	R	S	S	R	I	S	S	S	S	S	S	S	1	J	dt11af	11a
SP11	R	R	S	R	S	S	S	S	R	S	R	R	R	S	S	4	D	dt11a	11a
SP12	R	R	S	R	S	S	R	I	S	S	S	S	S	S	S	1	C	dt10h	ND
MSSP																			
SP13	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	0	A	ND	ND
SP14	R	R	S	S	S	S	S	S	S	S	I	I	S	S	S	0	I	ND	ND
SP15	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	0	A	ND	ND
SP16	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	0	G	ND	ND
SP17	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	B	ND	ND
SP18	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	0	E	ND	ND
SP19	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	0	D	ND	ND
SP20	R	R	S	S	S	S	S	S	S	S	R	I	S	S	S	1	F	ND	ND
SP21	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	0	L	ND	ND
SP22*	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	0	H	ND	ND

Amp, Ampicillin; Amx, amoxicillin; Cef, cephalothin; Chl, chloramphenicol; Cip, ciprofloxacin; Cli, clindamycin; Ctt, cefotetan; dct, *dru* cluster; Ery, erythromycin; Gen, gentamicin; I, intermediate; Mxf, moxifloxacin; ND, not determined; Ote, oxytetracycline; Oxa, oxacillin; Pen, penicillin; Rif, rifampicin; R, resistant; RS, resistance score; S, susceptible; Tet, tetracycline.

*Samples taken from same animal, 21 months apart.

†Samples taken from different sites in the same animal.

‡Samples taken from the same animal, 4 months apart.

Resistance scores ranged from 1 to 7 (mean 5.2, median 6) for the MRSP isolates and were 0 or 1 for the MSSP isolates.

Molecular characterization

Using the *Sma*I restriction enzyme, all 10 of the MSSP and three of the 12 MRSP isolates could be classified into 11 PFGE pulsotypes. MRSP isolate SP11 and MSSP isolate SP19 were assigned to the same pulsotype (pulsotype D). The nine MRSP isolates that could not be typed using *Sma*I had the same PFGE pulsotype (pulsotype K) using the *Cfr*91 restriction enzyme.

All 12 MRSP isolates were typable by *dru* typing (Table 2). Four different *dru* types were identified. The nine pulsotype K isolates belonged to *dru* type dt11cb. Single isolates of *dru* types d11af (dt11a-associated), dt11a and dt10h were also identified. Apart from dt10h, all of the *dru* types had been grouped into the 11a *dru* cluster in previous studies (Table 2). The dt11cb isolates were resistant to four to seven non-β-lactam antimicrobials (mean 6.2; median 6), whereas the three remaining MRSP isolates were resistant to one to four non-β-lactam antimicrobials (Table 2).

DISCUSSION

We report the first isolation of MRSP from dogs with recurrent pyoderma in Australia, with the first isolates obtained in February 2011. While it is entirely possible that MRSP isolates were present in Australia prior to this date, they were probably not recognized as, at the time, veterinary diagnostic laboratories were not routinely screening *Staphylococcus* isolates for oxacillin resistance. As reported by other groups (Sasaki *et al.*, 2007; Ruscher *et al.*, 2009; Perreten *et al.*, 2010), the majority of MRSP isolates in this study were found to be resistant to multiple antibiotic classes.

In the current study, MRSP isolates were definitively identified using a combination of *in vitro* resistance to oxacillin and detection of the *mecA* gene by real-time PCR.

Using *Sma*I, only three of the MRSP isolates were typable by PFGE, with each belonging to a distinct pulsotype. The nine MRSP isolates not typable by *Sma*I belonged to one unique *Cfr*91 pulsotype. In contrast, the 10 MSSP isolates belonged to nine different pulsotypes, indicating high heterogeneity. MRSP isolates that could not be resolved by *Sma*I PFGE were first reported in the Netherlands, where they were associated with ST29. These isolates could be typed by PFGE using *Cfr*91 (Laarhoven *et al.*, 2011). Most recently, a high proportion of atypical MRSP isolates were obtained from dogs and cats in Israel and Thailand, with the majority belonging to ST45 and shown to contain a novel SCC*mec* (ΨSCC*mec*$_{57395}$) (Perreten *et al.*, 2013). While the ST45 isolates from Israel were highly clonal and belonged to *dru* type 11cj, the 17 ST45 isolates from Thailand were more diverse and could be further subdivided into four *Cfr*91 pulsotypes and five *dru* types. Four isolates were identified as dt11cb. It is possible, therefore, that the nine Australian *Sma*I non-typable MRSP isolates obtained in our study are most closely related to the MRSP ST45 isolates from Thailand. However, a direct comparison using multilocus sequence typing will be required to confirm this hypothesis.

In the present study, *dru* typing showed that 11 of the 12 MRSP isolates belonged to *dru* cluster 11a. Although the 11a *dru* cluster has been previously reported to be associated with the internationally disseminated ST68 clonal lineage (Weese *et al.*, 2013), the recent findings of Perreten *et al.* (2013) confirm that it is not exclusively associated with this ST. The nine *Sma*I non-typable MRSP isolates that belonged to *dru* type dt11cb were highly resistant, whereas the three remaining MRSP cases with different *dru* and PFGE types had lower resistance scores. The last isolate was typed as dt10h, which appears to be an emerging MRSP clonal lineage in Canada (Weese *et al.*, 2012).

Nosocomial transmission might explain the cluster of five MRSP infections caused by dt11cb strains that occurred in four dogs within a 22-day period in 2011. However, bacterial cultures from samples from other dogs seen during this period by the dermatology clinic, as well as by other departments within the veterinary hospital, yielded only MSSP isolates. In addition, isolates SP6 and SP7 (obtained at the same time from different sites) were collected from a dog that presented for the first time to the clinic from a region about 1538 km from Perth. While the clinic adopts the practice guidelines recommended by the British Small Animal Veterinary Association (2011) for infection control, nosocomial transmission cannot be completely ruled out. However, the cluster of cases might just be a temporal association, given that some of the dogs originated from distinct geographical locations in Western Australia.

Current systemic antibiotics reported to be effective against MRSP are chloramphenicol, amikacin and rifampicin (which should always be given in combination with another class). Each of these antibiotics is frequently associated with undesirable adverse events, potential toxicity and/or expense (Frank & Loeffler, 2012; Papich, 2012). The dt11cb isolates recovered in the current study were resistant to chloramphenicol, thus further limiting treatment options for these cases. All MRSP and MSSP infections in this study resolved after treatment with topical antimicrobials (e.g. 3 % chlorhexidine, 2 % mupirocin, 2 % fusidic acid), with or without concurrent systemic antibiotics (e.g. rifampicin, chloramphenicol) selected on the basis of *in vitro* antimicrobial susceptibilities. To avoid relapses, the underlying diseases were also managed appropriately. Given the *in vitro* susceptibility of the isolates to moxifloxacin and resistance to ciprofloxacin, combined therapy with dual-targeting fluoroquinolones such as pradofloxacin (Wetzstein & Hallenbach, 2011) or moxifloxacin and other antimicrobial classes to which MRSP isolates are susceptible could be an appropriate systemic approach for deep pyoderma caused by MRSP, to prevent the rapid emergence of resistance in either class.

Meticillin resistance is conferred by the *mecA* gene, which encodes a modified penicillin-binding protein (PBP2a) with low affinity for all β-lactam antibiotics (penicillins, cephalosporins and carbapenems), rendering them ineffective despite apparent *in vitro* susceptibility to some β-lactams. It is therefore important that veterinary diagnostic laboratories include screening for oxacillin resistance in their routine susceptibility testing for coagulase-positive *Staphylococcus*.

In conclusion, we report the isolation of MRSP from dogs with chronic recurrent pyoderma referred to a specialist dermatology practice in Perth, Western Australia. The first nine MRSP isolates, which appeared to be clonally related on the basis of *Cfr*91 PFGE and *dru* typing, possessed a multidrug-resistant (resistant to more than three antibiotic classes) phenotype. Similar MRSP isolates with the same *dru* type and a novel SCC*mec* element have recently been reported in another country within the Asia-Pacific region. The spread of dt11cb PFGE pulsotype K MRSP isolates in the rest of Australia remains to be determined.

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